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# Immunization Procedures for *E. coli* Proteins

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## ABSTRACT

Three immunization procedures were compared for the production of antibodies to the minor components of a complex *E. coli* protein (ECP) mixture: a conventional protocol and two methods that allow for the selective in vitro (cascade) or in vivo (passive) depletion of highly immunogenic proteins. An indirect ELISA showed that a maximum ELISA antibody titer was obtained with all the procedures 60 d after immunization. Analysis of these antisera by two-dimensional SDS-PAGE immunoblots, however, demonstrated that antibody reactivity to minor components in the mixture was not achieved until 112 d. This analysis also showed that a marked improvement in antibody response to minor components was obtained with the cascade immunization procedure. The mean titer and spectrum of antibody reactivity was similar for each group, and suggested that, although some individual variation was noted, the improvements observed were the result of the protocol used. Thus, for these ECPs, and multiple antigen mixtures in general, the preferred immunization protocol should employ at least three hosts and utilize the cascade immunization of Thalhamer and Freund (*1*). Characterization of the resulting antisera is best performed by use of silver stained two-dimensional SDS-PAGE and immunoblotting.

**Index Entries:** *E. coli* protein; cascade immunization; two-dimensional immunoblotting; antigenic competition; rDNA product purity.

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## INTRODUCTION

The production and characterization of antisera to protein mixtures is increasingly important in areas such as the immunochemical characterization of allergen extracts (*2*), immunoblot fingerprints of hospital pathogens (*3*), and the development of bacterial vaccines (*4–6*). In addition, we have reported the use of complex antisera against *E. coli* protein (ECP) mixtures as a reagent for immunoassays designed to detect host cell impurities in rDNA produced pharmaceuticals (*7*). The detection and accurate quantitation of ECPs in these assays requires a condition of antibody excess for each protein to allow the observation of a linear dilution response. The acquisition and characterization of broad spectrum antisera against bacterial protein mixtures, therefore, is a fundamental goal in these areas.

The production of such complex and broad spectrum antisera presents two problems. First, immunization with ECP mixtures may be complicated by the distribution of proteins in the mixture, the particular properties of each component, or the host response, which may be dominated by antigens in high concentration or by those that are strongly immunogenic. Also, whereas the evolutionary distance of *E. coli* from mammals might suggest that all *E. coli* proteins would be strong immunogens, examples of shared antigenic determinants are known, and it is possible that tolerance to these determinants may occur.

Two membrane proteins of *E. coli* share at least one antigenic determinant with the alpha 1-subunit of the acetylcholine receptor (*8*), and material from *E. coli* is reactive with antibodies against insulin (*9*), brain gangliosides (*10*), and chorionic gonadotropin (*11*). Finally, antigenic competition has been demonstrated as a complicating factor in immunization procedures using mixtures of *E. coli* proteins (*1,12*).

Second, methods for the characterization of complex antisera are difficult. Antisera to *E. coli* protein mixtures have been developed with impressive spectra of reactivity using conventional immunization methods (*7,13,14*). An exact assessment of the spectrum of antibody reactivity is often limited, however, by the resolution of the analytical methods used. Counter immunoelectrophoresis (CIE), as used by Owen or Thalhamer and Freund, is advantageous in that antibody–antigen interactions occur under native conditions and the relative amounts of antibody and antigen can be studied. This system, however, is limited by the relatively low sensitivity of detection and resolution for complex mixtures of reacting species. One-dimensional silver stained SDS-PAGE and immunoblotting used in both our study and by others provides sensitive detection limits but lacks resolution. Therefore, improved characterization methods that have a high degree of resolution and sensitivity are required to best compare potential improvements in the production of these complex antisera.

Recently, two immunization procedures designed to enhance the immune response to multiple antigen mixtures have been reported. The

cascade immunization technique (*1*) utilized in vitro depletion of ECPs that had previously elicited an antibody response by absorption of these dominant immunogens with antibodies obtained from an earlier antiserum. The passive immunization procedure (*12*) relied on in vivo blocking of strong immunogens by concurrent administration of antiserum obtained previously with the ECP mixture. This latter report demonstrated the presence of an apparently poorly immunogenic ECP to which a humoral response could only be elicited by this passive procedure.

Given the potential of these methods to improve the immune response to multiple antigen mixtures, we describe here a comparison of these two methods to a conventional immunization procedure reported previously (*7*). This study also extends the previous reports by providing a detailed characterization of the component ECPs and the subpopulations used for subsequent immunizations. Further, various methods for antisera characterization are also evaluated, including the use of two-dimensional electrophoresis and immunoblotting as a tool to examine the production of antibodies to minor components of the ECP mixture.

## MATERIALS AND METHODS

### Preparation of ECPs

The ECPs were prepared with cell paste from *E. coli* K-12, W3110 containing the plasmid pBR322. The cell paste was processed by ammonium sulfate precipitation and ion exchange chromatography to mimic typical purification process steps. The ECPs were dialyzed into PBS and protein concentration determined by Bradford protein assay using BSA as a standard.

### One-Dimensional Gel Electrophoresis

The ECP preparation or subsets of the preparation were analyzed on 12.5% SDS-PAGE, as described by Laemmli (*15*). After electrophoresis, the gels were either stained with Coomassie brilliant blue or silver (*16*).

### Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed using a modification of the methods of O'Farrell (*17*) and Garrels (*18*). For first dimension isoelectric focusing (IEF), an ampholyte mixture of 70% pH 4–6.5, 15% pH 5–8, and 15% pH 3–10 gave optimal resolution of the ECP minor components. The ampholyte mixture was incorporated into a final mixture of 4% acrylamide, one % Triton X-100, 2% ampholytes and 8 *M* Urea. After the addition of 7 μL of 10% APS and 2 μL TEMED per 1.5 mL of the IEF mixture, the solution was added to 1.5×180 mm glass tubes, sealed

at one end, and allowed to polymerize for 1.5 h. The gels were then prefocused to establish the pH gradient by running to 1000 V with the current and wattage limiting in a tube gel apparatus using 0.1 *M* NaOH in the upper chamber and 0.01 *M* $H_3PO_4$ in the lower chamber.

The ECP sample (30 μg) was warmed to 37°C and focused for 14,000 V-h at 25°C. The tubes were placed on ice and the gels extracted by air pressure. The acidic end was marked with charcoal black and the gels equilibrated with sample buffer. The tubes were then firmly placed on the second dimension slab gel (12% SDS-PAGE) and run at 50 mAmps until the dye front reached the bottom on the slab.

## Immunoblot Analysis

Immunoblotting was performed according to the method of Towbin et al. (*19*). For one-dimensional gels, the electroelution time from the gel to nitrocellulose was 40 min at one Amp, whereas transfer of 2 D gels was conducted for 1.5 h at one Amp. The sheets were trimmed to match the gels and blocked with NET-gelatin buffer, pH 7.2 (50 m*M* Tris, 5 m*M* EDTA, 150 m*M* NaCl, 0.05% Triton X-100, and 0.25% gelatin).

The sheet or strips of the sheet were incubated with the rabbit antisera for 2 h at RT, washed extensively with NET-gelatin, and incubated with Protein A conjugated to horseradish peroxidase (Tago, Inc., South San Francisco, CA) for enzyme immoblots or $^{125}I$ Protein A (Amersham, Arlington Heights, IL) for autoradiography. The enzyme immunoblots were washed again with NET-gelatin, rinsed with PBS containing 0.05% Tween 20, and exposed to substrate, according to the method of Adams (*20*). Autoradiograms were also washed with PBS-Tween 20, blotted dry, and exposed to X-ray film (Kodak X-OMAT-AR). Optimal exposure time was determined by the intensity of the spots on the film.

## Indirect ELISA

The ELISA procedure was performed as follows: microtiter plates (Nunc, Kamstrup, Denmark) were coated with 100 μL per well of 5 μg/mL ECPs in 0.01 *M* carbonate buffer, pH 9.6, overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and then excess sites blocked with 200 μL/well of PBS containing 0.1% gelatin and 0.05% Tween 20 (PBSGT) for 1 h.

After washing, 100 μL of antisera was diluted in a serial twofold manner across the plate and incubated for 1 h at RT with agitation. The plates were washed and 100 μL of Protein-A conjugated to horseradish peroxidase (Tago, Inc.) added to the wells for 1 h. After washing, each well received 100 μL of O-phenylenediamine substrate solution (40 μL 30% $H_2O_2$, 40 mg OPD, 100 mL 0.15 *M* phosphate-citrate buffer, pH 5.0) for 10 min at ambient temperature. The reaction was stopped with 100 μL per well of 4.5 N $H_2SO_4$. The resulting absorbances were read at 492 nm using

a Titertek plate reader (Flow Labs, Mclean, VA) and the antisera titer calculated as the log of the reciprocal dilution, which resulted in a 50% maximal signal.

## Immunization Procedures

Conventional Immunization Procedure: One-half mg of ECPs were administered to each of three female New Zealand White rabbits subcutaneously along 5 to 10 sites on the back. The primary injection was administered in Complete Freunds Adjuvant (CFA) on d 1 and a second injection in Incomplete Freunds Adjuvant (ICFA) on d 7. Thereafter, the rabbits were bled (30 mL per rabbit) 7 d after each boost, and subsequent injections (0.5 mg per rabbit) were given 7 d after each bleed. The serum was retained and stored frozen (−20°C) until the time of analysis.

Passive Immunization Procedure: The passive immunization procedure was based on a modification of the method of Thalhamer and Freund (*12*). Briefly, 3 rabbits received primary and subsequent injections as described for the conventional immunization procedure. The injections of antigen following the primary inoculation, however, also included a concurrent intravenous injection of 0.5 mL of serum obtained from the individual rabbit 7 d earlier. This protocol was followed until week 12, at which time the antigen injections were given intramuscularly without ICFA in an effort to increase the amount of immunogen in the circulation and thus its exposure to the passively administered antiserum.

Cascade Immunization Procedure: The cascade immunization procedure was based on a modification of the method of Thalhamer and Freund (*1*). Three rabbits received a primary injection of ECP in CFA and a subsequent injection in ICFA as described in the conventional immunization procedure. A serum sample was taken from each animal 7 d later. The antisera from the 3 rabbits were pooled (45 mL sera total) and the crude immunoglobulin fraction (crude Ig) was obtained by the addition of saturated ammonium sulfate (SAS), pH 7.2, to a final concentration of 40% according to the method of Good et al. (*21*).

The crude Ig preparation was then dialyzed against PBS overnight (3 changes of 2 L each) and coupled to Affi-gel 10 (Bio Rad Laboratories, Richmond, CA). The coupling of the crude Ig was greater than 90%, as determined by absorbance of the reaction supernatant at 280 nm.

After extensive washing of the column in PBS followed by 0.2 *M* glycine-HCL, pH 2.4, the column was reequilibrated in PBS. A sample containing 4.5 mg of the ECP was chromatographed on the column and fractions were collected. Fractions containing protein were adjusted to a concentration of 5 μg/40 uL (assuming an extinction coefficient of 1.0 for the ECP preparation) and examined by silver stained SDS-PAGE gels. Fractions that were depleted of one of more ECPs (compared to the starting preparation) were used for the subsequent immunization injection (*see*

Results, Fig. 2). This procedure of ECP adsorption was repeated with subsequent antisera obtained on d 28 and 42 (Fig. 2B, C) and the resulting depleted ECP fractions used for injections on either d 35 or 49 and 62, respectively. The concentration of ECPs was 0.1, 0.25, 0.25, and 0.25 mg/rabbit for the injections administered on d 21, 35, 49 and 62, respectively (Fig. 1). Thereafter, the rabbits received injections as described in the normal immunization procedure.

## RESULTS

### Characterization of the ECP Preparation by Coomassie Blue Stain

To determine the approximate distribution of the component proteins within the ECP preparation, a Coomassie brilliant blue stained gel was analyzed by laser densitometry. The reduced ECP preparation is composed of 3 major species, 2 (approximately 66% of the total) in the MW range 40–60 kD and 1 (14.6%) at approximately 20 kD (Fig. 2). Each of the other bands in the mixture was individually less than 4% of the total. Seven bands represented less than 1%, of which 2 represented 0.2% of the total.

### Selection of Immunogens for Cascade Procedure

Figure 3A, lane 3 shows the content of the ECP depleted fraction obtained after immunoadsorption with sera taken at d 14. It is apparent that certain proteins had been adsorbed by the immobilized antibody (Fig. 3A, lane 3, arrows). This fraction was used as the immunogen for the subsequent injection as shown in Fig. 1. This method of immunoadsorption was repeated with antiserum obtained on d 28 and 42 (Fig. 1). The silver stain SDS-PAGE analyses of the early flowthrough fractions are shown in lane 3, Fig. 3B (d 28 antiserum) and 3C (d 42 antiserum). Examination of these silver stained gels of the ECP fractions (all at equal protein loads) demonstrated continued depletion of individual ECPs by the immunoadsorption procedure. The ECP fraction represented by Fig. 3B, lane 3 was used as the immunogen for the d 35 injection, and the ECP fraction represented by Fig. 3C, lanes 3 and 4 was used as the immunogen for the injections on d 49 and 62.

### ELISA Determination of Total Antibody Concentration

Determination of the antibody titer was made by an indirect ELISA method. The antisera for each group were prepared from equal volumes of serum from each animal. Baseline sera did not possess endogenous antibodies to the ECPs and the d 14 group titers were similar in all groups,

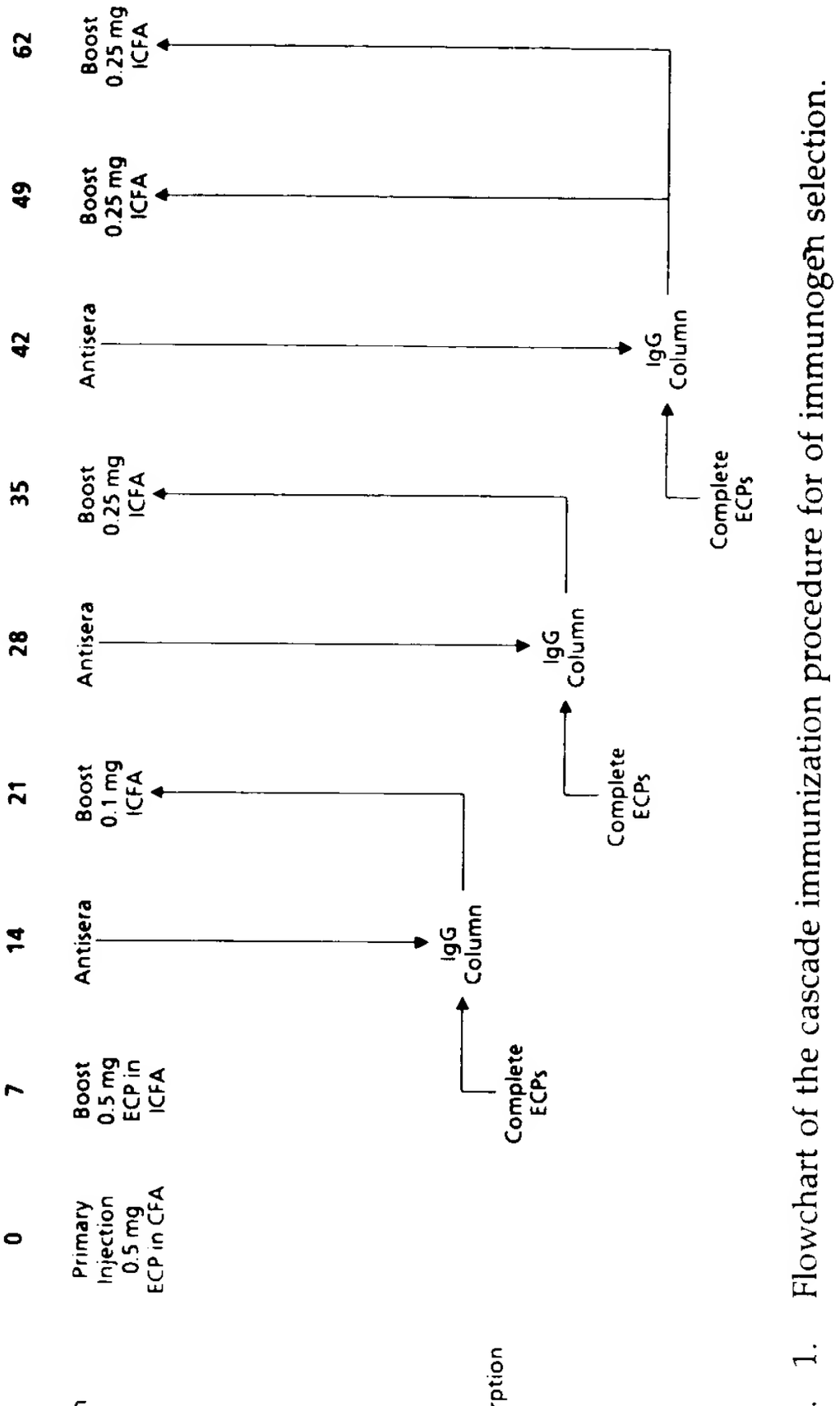


Fig. 1. Flowchart of the cascade immunization procedure for of immunogen selection.

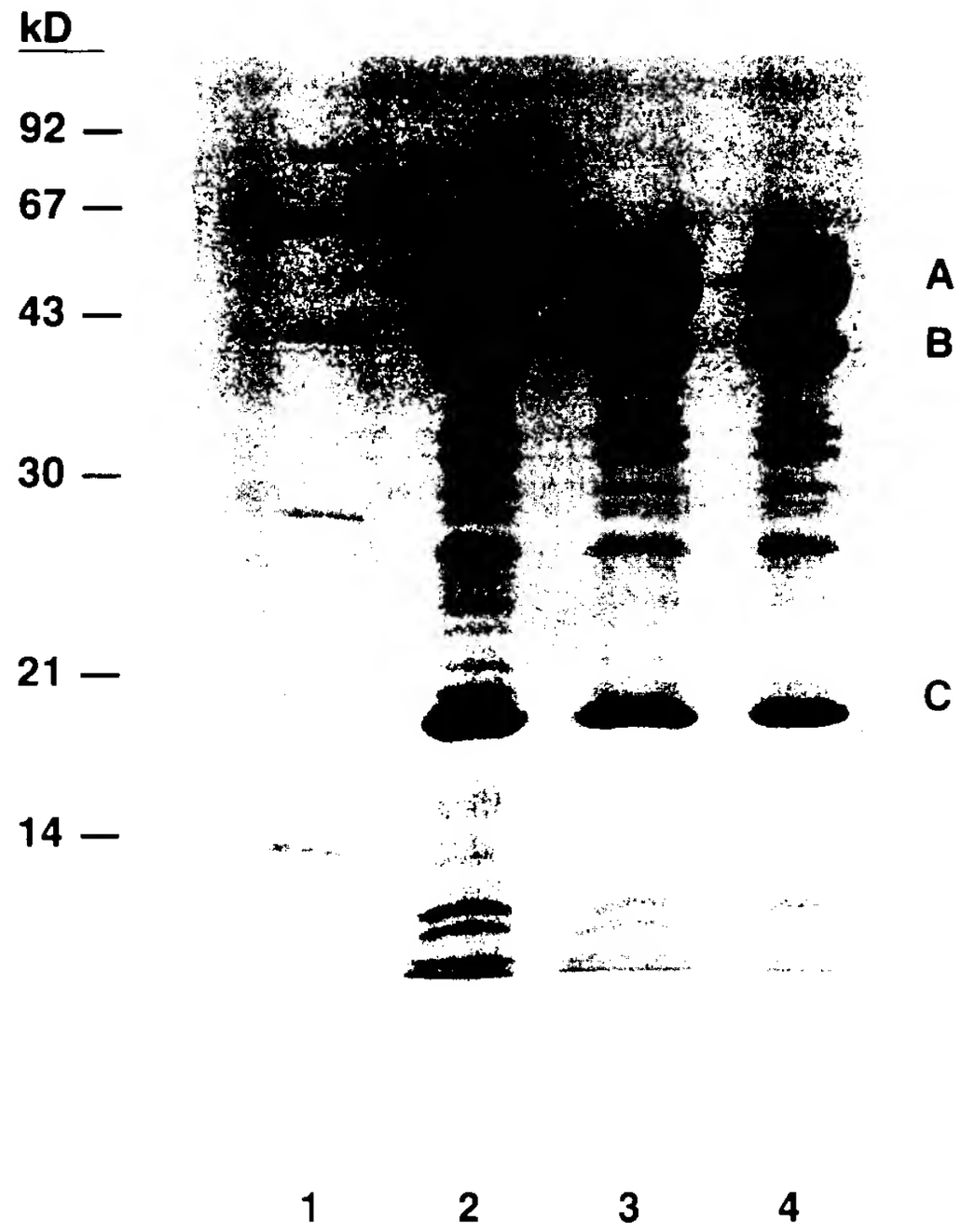


Fig. 2. Distribution of the component ECPs on 12.5% SDS-PAGE after reduction and staining with Coomassie blue dye. Bands A, B, and C represent approximately 80% of the total band area. Lane 1, standards of indicated MW. Lane 2 to 4, ECPs at 80, 60, 40 $\mu$g, respectively.

which was prior to any manipulation of the immunogen (Fig. 4). Examination of the early antibody response (through d 56) showed that the most rapid initial increase in titer was obtained with the conventional immunization protocol group. The passive immunization group was characterized by a depressed titer through d 42 and, to a lesser extent, through d 56. In general, however, after d 56 comparable titers were obtained, which suggested that the groups had achieved a maximum titer by d 60. The reason for the decrease in titer of the passive immunization group on d 140 is unknown.

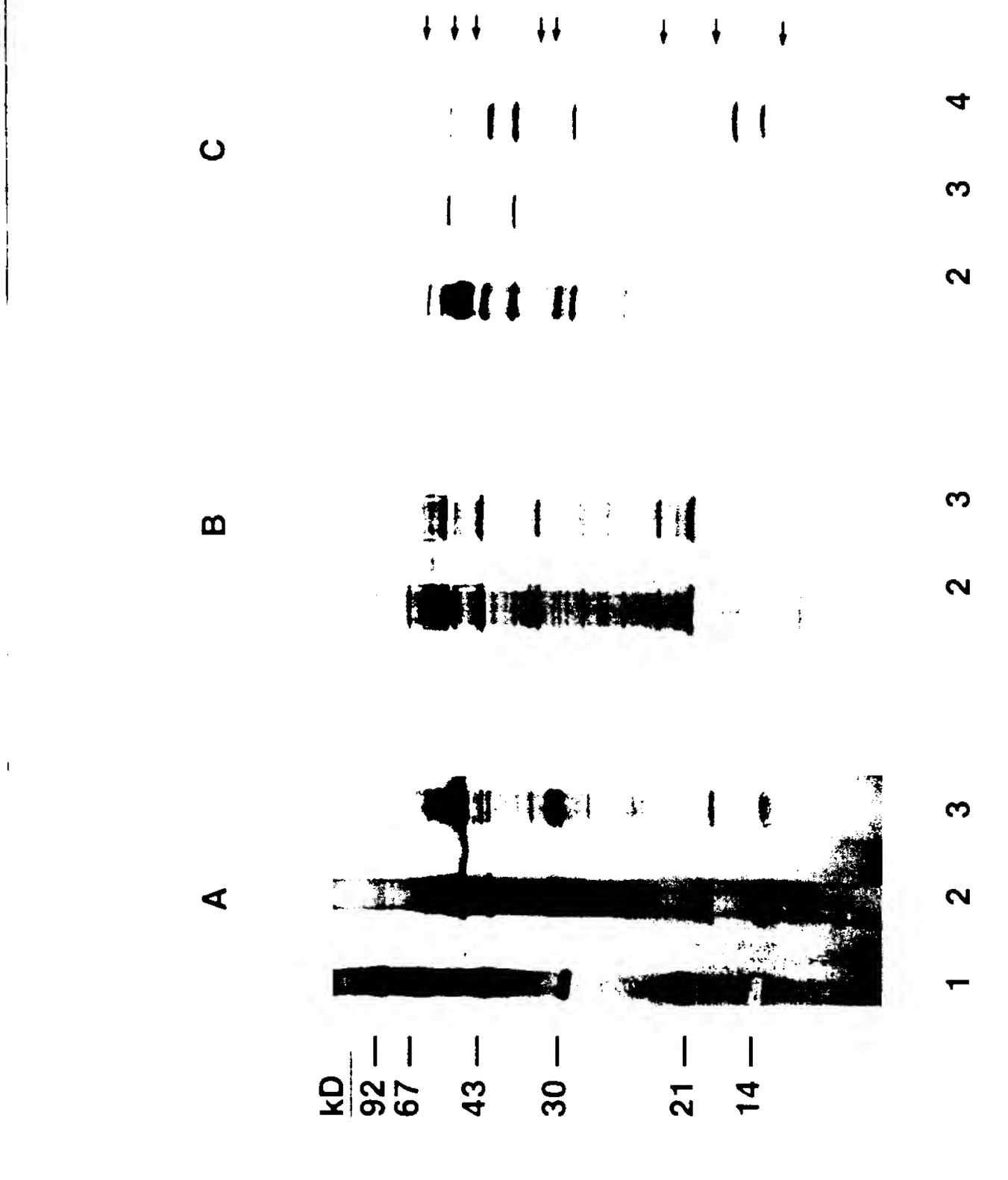


Fig. 3. Selection of ECP subpopulations for progressive iterations of the cascade procedure by silver stained SDS-PAGE. A: lane 1 MW standards, lane 2; column load, lane 3; column flowthrough fraction used for day 21 injection. B: lane 2; column load, lane 3; flowthrough fraction used for day 35 injection. C: lane 2; load, lane 3 and 4; fractions used for day 49 and 62 injections. Arrows show ECPs immunodepleted by early antibodies. It is apparent that a protein of approximately 50 kD has saturated its respective antibody and has begun to flow through the column (Panel C, lane 4).

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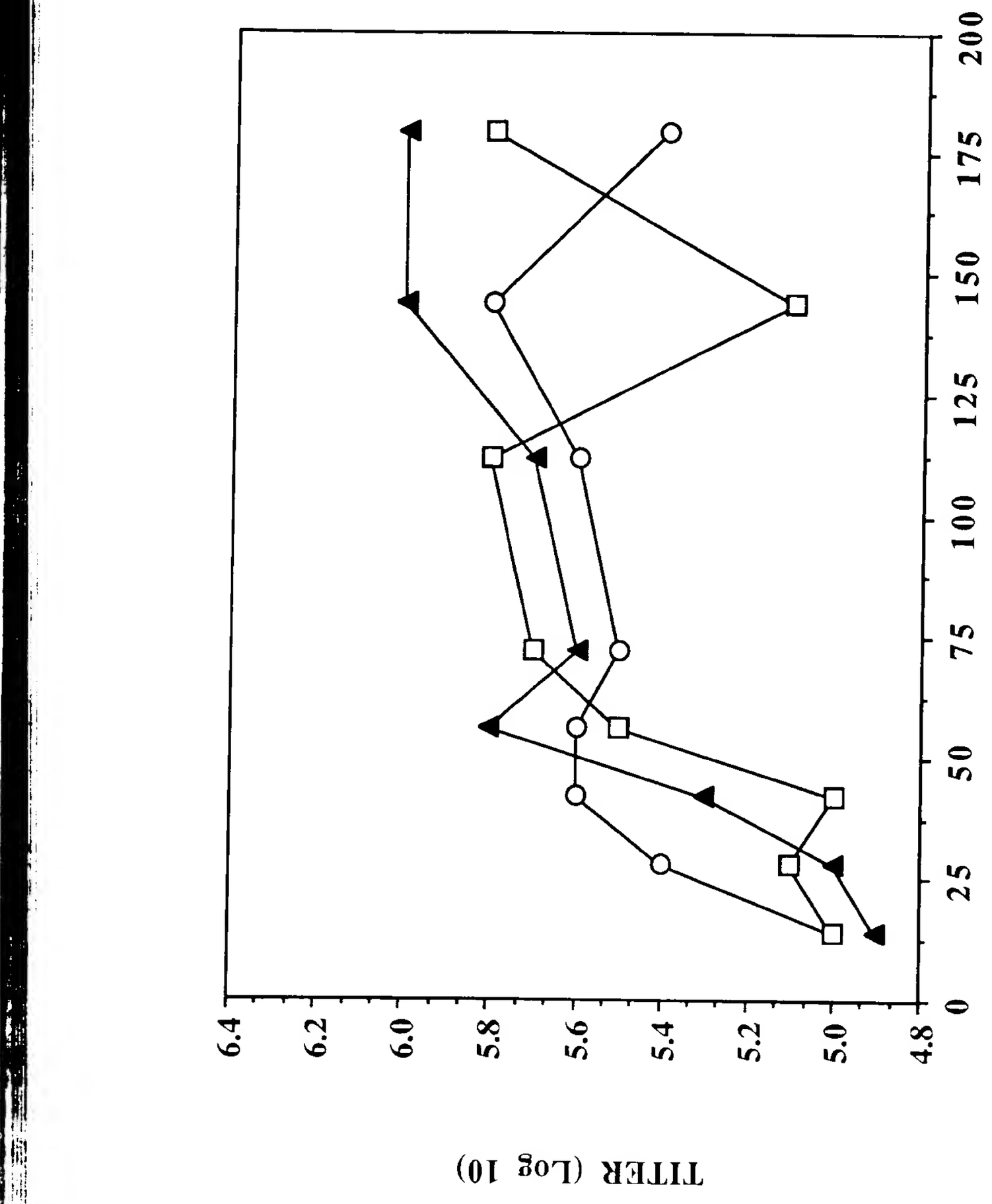


Fig. 4. Progression of group titers as determined by ELISA. The baseline titer for each of the group antisera was <1.0. (○): Conventional group antisera. (▲): Cascade group antisera. (□): Passive group antisera.

To assess the amount of individual variation within the groups, serum obtained from each rabbit at d 56 was also titered. Although differences were detectable between individual rabbits, the range and mean group response were similar and suggested that the group antisera characterization was a reasonable approach to the assessment of the immunization protocols (data not shown).

## Examination of Individual Host Response by Immunoblot Analysis

To examine the antibody response to components of the ECP mixture, the d 72 antiserum from each host was used for immunoblot analysis at a 1:10 dilution (Fig. 5). In general, the response to components above 21 kD was similar, although among lower MW proteins variation was notable between the individual hosts. This variation did not appear to be related to any particular immunization group, suggesting that results obtained with group antisera would not be biased by an individual high responder, nor to any particular low MW component. This was not a result of differential protein transfer, since all of the strips were cut from the same sheet of nitrocellulose. Perhaps these lower MW components contained fewer potential antigenic sites, which made subtle individual variation in this polyclonal system more apparent.

## Two-Dimensional Electrophoresis and Immunoblotting

To obtain additional resolution of the component ECPs, two-dimensional electrophoresis followed by silver stain or immunoblotting was performed. A first dimension IEF gel predominantly composed of pH 3 to 6.5 ampholytes resolved the ECP mixture into approximately 100 individual spots with major components that appear to match those at 40–60 kD and 20 kD observed in the one-dimensional gels (Fig. 6A). Initially, the d 56 antisera were selected for 2D immunoblots because the group ELISA titers had reached a plateau and the cascade and passive immunization groups had received at least 3 iterations of immunogen selection or passive administration of antibodies, respectively. Immunoblots performed with these antisera, however, detected only half of the minor components apparent by silver stain (data not shown).

To determine if a wider spectrum of antibodies was achieved later in the protocol, the d 112 antisera was used for immunoblotting (Fig. 6). These antisera were selected because the ELISA titers were again comparable and the cascade immunization group had received 3 injections of the complete ECP mixture at the same dose (0.5 mg) as the conventional immunization group. Also, the passive immunization group had received 2 administrations of the ECP mixture intramuscularly without adjuvant in an effort to increase the exposure of the ECPs to the passively administered antibodies. The immunoblots demonstrated an increase in the spectrum

of the group antisera was ~1:0. (○): Conventional group antisera. (□): Passive group antisera.

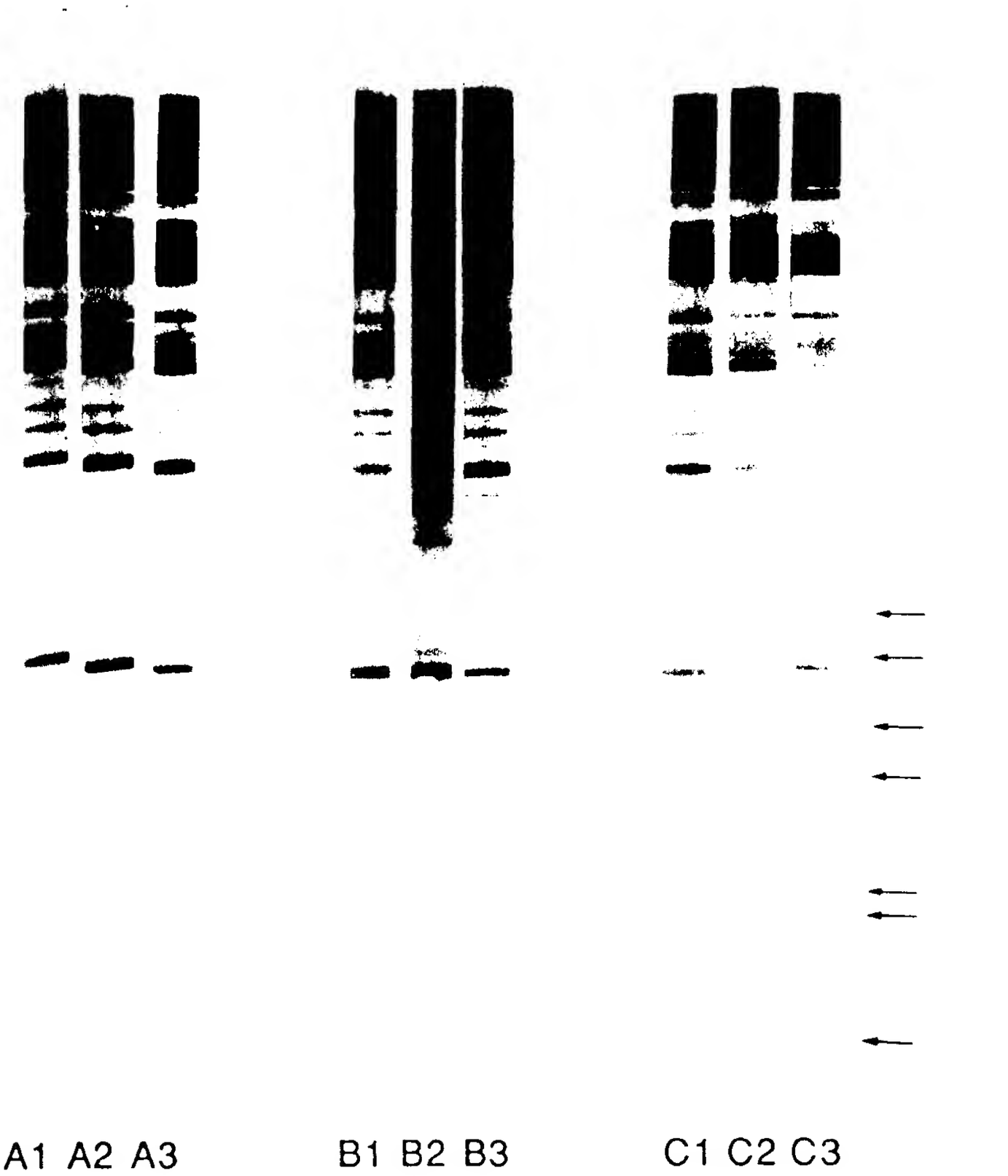


Fig. 5. Individual host variation by one dimensional immunoblotting. Conventional immunization group: A1, A2, A3. Cascade immunization group: B1, B2, B3. Passive immunization group: C1, C2, C3.

of antibody reactivity with each of the group antisera between d 56 and 112 and was primarily directed to minor components of the ECP mixture.

The cascade immunization antisera detected a number of minor component ECPs (indicated by the arrows in Fig. 6C) that were not observed with the conventional or passive antisera (Fig. 6B, D). It was quite clear from these data that the cascade antisera was far superior in its spectrum of antibody reactivity and, in fact, was comparable or superior in detection of ECPs to silver stain (Fig. 6A). The silver stain did, however, appear to have an improved detection of certain low MW or basic proteins.

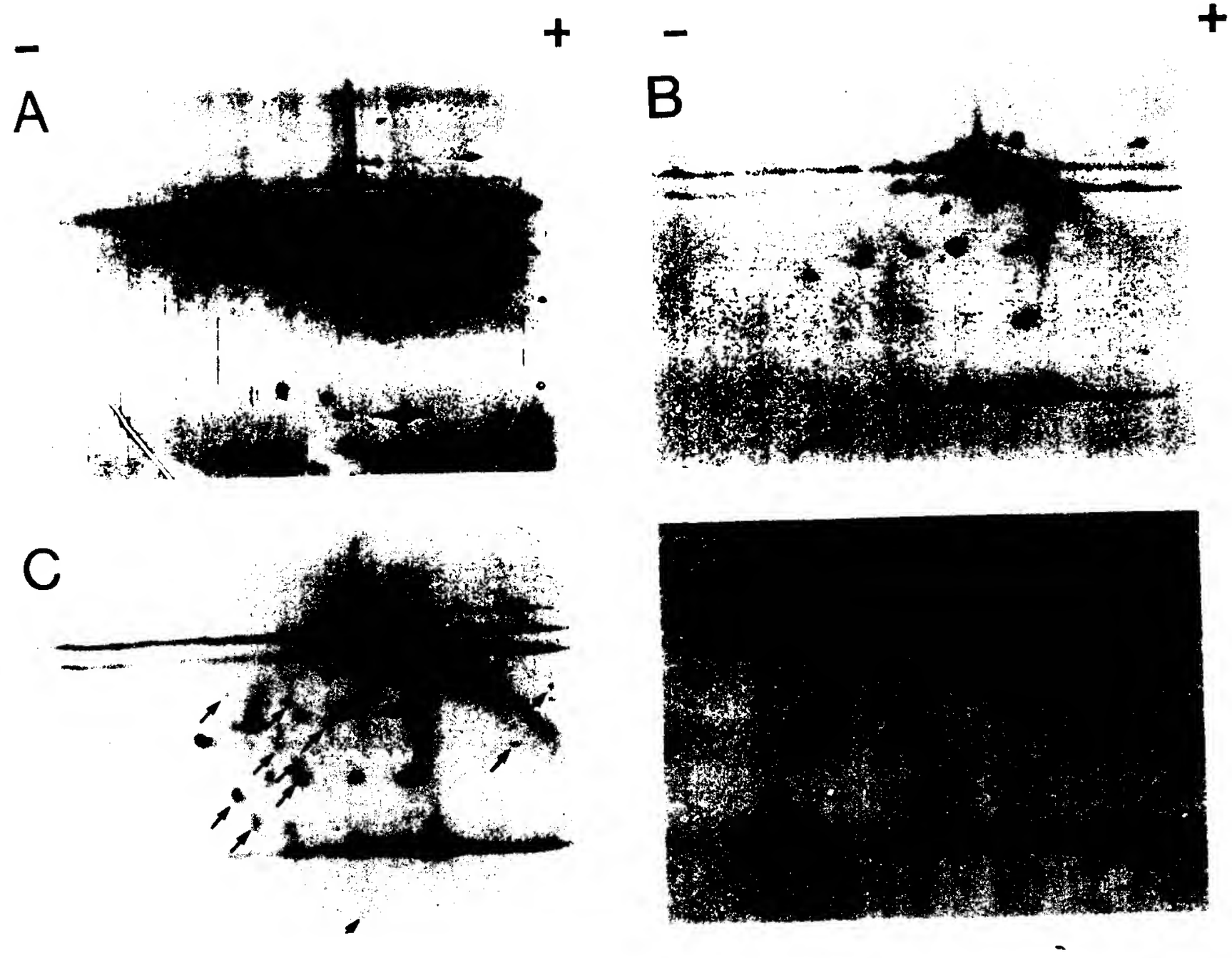


Fig. 6. Separation and detection of ECPs by two-dimensional gel electrophoresis. A: Silver stained. B: Immunoblot with conventional procedure day 112 antisera. C: Immunoblot with cascade procedure day 112 antisera. D: Immunoblot with passive immunization procedure day 112 antisera. Exposure time was 24 h.

Exposure of the blots to X-ray film for 48 h to increase sensitivity showed that a larger number of additional spots could be detected (Fig. 7). This increased spectrum of reactivity included many of the low MW and basic components observed with silver stain and may reflect lesser immunogenicity or differential transfer of these proteins. Also, many spots that were detected only by the cascade antisera at 24 h could be detected by the conventional protocol antisera at 48 h. This suggested that the differences in reactivity (seen in Fig. 6) were the result of differences in antibody titer. The spectrum of reactivity obtained with the passive procedure antisera exposed for 48 h remained markedly inferior (data not shown).

Different reactivity of antisera were not the result of differential transfer of proteins because the same sheet of nitrocellulose was used for all of the blots (after washing with acid between experiments). Exposure of the acid washed sheets did not reveal the presence of non-acid elutable antibodies because the addition of $^{125}I$ labeled protein A without primary antisera did not show any spots after a 24-h exposure (data not shown). Also, proteins were not eluted from the sheet because reblotting with the cascade antisera demonstrated the identical pattern after blotting had been performed with each of the group antisera (data not shown).

These data suggested that a mechanism of early priming by the cascade procedure resulted in a broader spectrum of antibody reactivity. This improvement, however, also required additional time (56 d) and/or subsequent injections of the total antigen mixture. The priming with poorly immunogenic ECPs may have allowed an early induction of a response to the poor immunogens that ultimately resulted in a higher titer antibody response in this group.

## DISCUSSION

This study has demonstrated that high titer, broad spectrum antisera can be developed to an *E. coli* protein mixture in 112 d. Of the 3 immunization protocols examined here, a clear benefit was achieved by use of the cascade immunization method of Thalhamer and Freund (*1*). This benefit was characterized by the improved production of antibodies to minor components in the antigen mixture. This study also demonstrated that the examination of the antibody response to ECP mixtures must be rigorous in nature. The ELISA method indicated that plateau titers were reached for each of the three immunization groups in 60 d and with comparable titers. Yet, examination of the response by two-dimensional electrophoresis followed by immunoblotting demonstrated that broad spectrum antisera (comparable in ECP detection to silver stain) was not obtained until 112 d into the study. The ELISA signal was probably dominated by the major proteins and was not able to detect large changes in concentration of antibodies to the minor proteins.

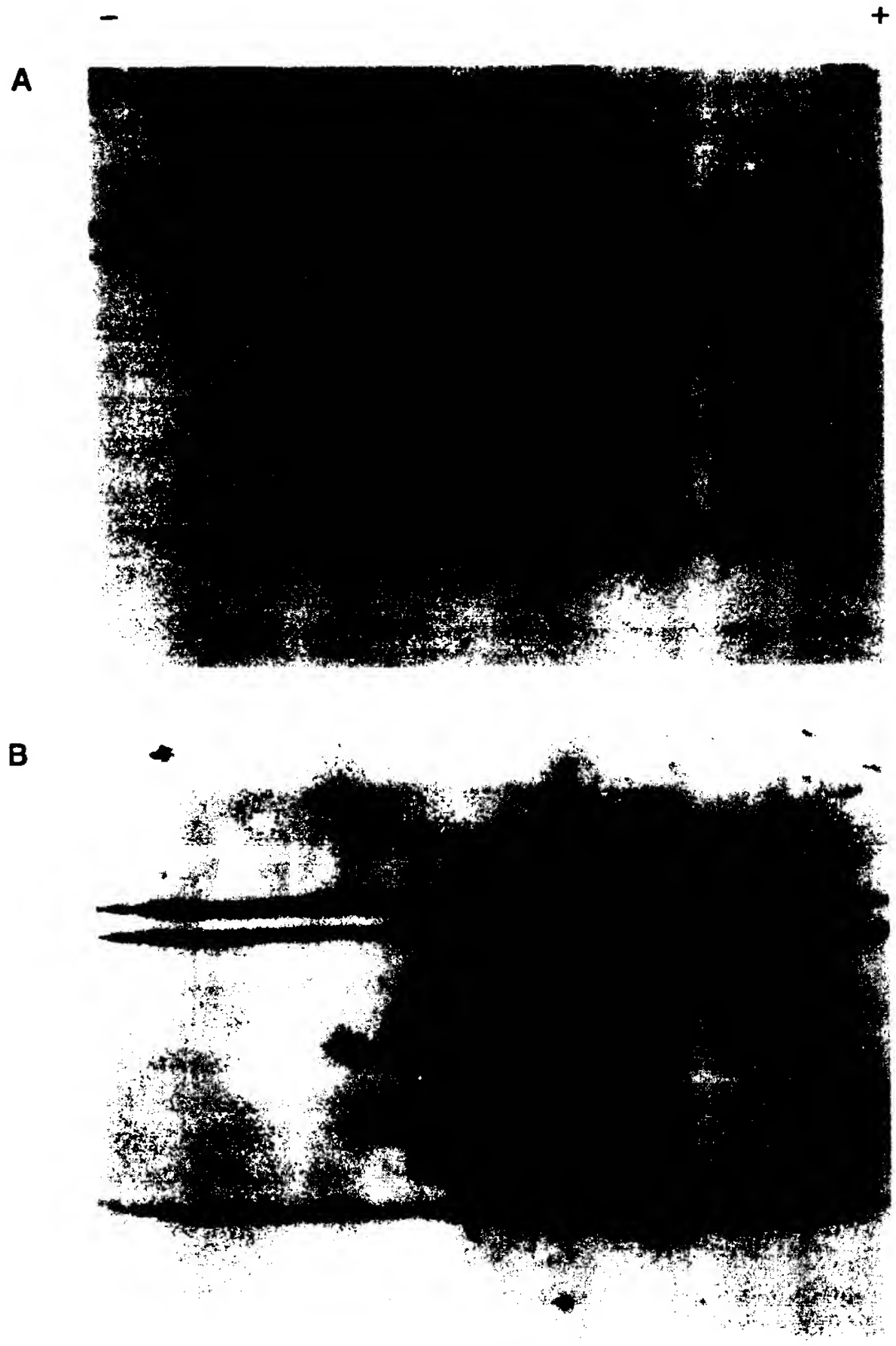


Fig. 7. Immunoblots with day 112 conventional antisera (A) and cascade antisera (B), 48-h exposure.

Therefore, characterization of the antigen mixture, along with the group and individual antibody response, is required for the development of comprehensive antisera to multiple antigen mixtures. In our study, the most informative methods for this analysis were two-dimensional gel electrophoresis and immunoblotting.

A number of interesting observations arose from this study. First, it was apparent that the greatest individual variation was observed in the

response to proteins of low MW (10–21 kD) (Fig. 5). It is certainly expected that the number of potential antigenic sites would decrease with a decrease in MW, but this amount of variation to components of relatively large size (> 10 kD) was surprising using polyclonal antisera. This is because the production of only 1 antibody to an antigenic determinant is theoretically sufficient to mask unresponsiveness to other determinants. The observation of this individual variation does emphasize a critical parameter for immunization with multiple antigen mixtures. A number of animals should be immunized to avoid possible variations in host response. The complex nature of multiple antigen mixtures essentially requires the animal to be a high responder to each antigen in the mixture, which is unlikely. The importance of using many animals for immunization has been described (*22*), but it is common for reports to use only one or an unspecified number of animals (*7,14,23*). It is clear from the data present here (Fig. 5) that at least 3 animals were required to obtain a comprehensive spectrum of antibodies to the low MW components in this mixture regardless of the immunization protocol used.

The improved response with the cascade protocol was interesting. Perhaps the change in the relative concentrations of the component proteins had a significant effect on antigenic competition. The initial steps of antigen presentation between macrophages and T-cells have been shown to be HLA restricted and recent studies have demonstrated this restriction to occur on at least 1 level by the binding of peptide antigens to MHC class II on antigen presenting cells (*24,25*). Further, although a single MHC class II molecule has been demonstrated to bind many distinct peptides, only a single peptide binding site exists and the binding of dissimilar peptides is competitive for that site (*24,25*). These findings raise interesting questions regarding multiple antigen mixtures and the improved response to the minor components observed with the cascade immunization protocol. Assuming antigen processing and presentation is indiscriminate, it would be reasonable to assume that those antigens in the highest local concentration would be presented most frequently. Competition for the binding site on a given MHC class II molecule would be expected to amplify this local bias for peptides present in higher concentrations. The depletion of component proteins in the cascade protocol may have alleviated this bias toward proteins in the highest concentration and allowed a more favorable probability for presentation of proteins that previously were in concentrations too low to effectively compete for MHC class II binding sites. Indeed, the proteins depleted from the mixture during the immunoadsorption steps of the cascade procedure would appear to be those in the highest concentration as assessed by Coomassie brilliant blue staining. For example, the third iteration of the cascade procedure (Fig. 4C) resulted in an antigen mixture largely devoid of the component at approximately 60 kD, which comprised approximately 42% of the total mixture. Presumably, this may have resulted in the enhanced response to the

minor component proteins observed in the two-dimensional immunoblots with the antisera from the cascade protocol rabbits.

This model of competition for MHC class II binding would seem to fit the results obtained in this study. The use of the cascade procedure may have facilitated the stimulation of specific T cells early in the immunization procedure, and, perhaps, resulted in a longer period of time for B cell proliferation or improvement in antibody affinity. This progression of antibody affinity may occur primarily by somatic mutation, which is time and antigen exposure dependent (*26*).

In general, the passive immunization procedure resulted in an inferior antibody response to the ECPs. The principle behind this procedure was that antibodies would block major ECPs or perhaps that antibody-ECP complexes would be cleared rapidly and allow minor components to become "Major" components. The role of antibody in modulating the immune response is well known and may occur by several mechanisms (*27*). Antibody can concentrate antigen on the surface of macrophages via Fc receptor binding, analogous to a mechanism proposed for B cell antigen presentation (*28*). A mechanism of this type may also explain the poor response observed with the passive group to other minor components in the ECP mixture. If the antibody-ECP complexes, instead of being rapidly cleared, actually stimulated the processing and presentation of antigen, then the administration of passive antibodies would be expected to further enhance the response to strong immunogens rather than avoid antigenic competition.

A final comment should be made regarding these studies. First, although the 3 animals in each protocol comprise a relatively small group, the data presented here suggest that the group response was similar despite some individual differences. Nonetheless, the improved results with the cascade procedure might have been the result of chance owing to a given rabbit's response. A study of the cascade procedure using larger groups of animals would be required to address this question directly.

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